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Short communication

High genetic diversity in field isolates of *Trypanosoma theileri* assessed by analysis of cathepsin L-like sequences disclosed multiple and new genotypes infecting cattle in Thailand

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ABSTRACT

In this study, we describe the first survey in Thailand of *Trypanosoma theileri*, a widespread and prevalent parasite of cattle that is transmitted by tabanid flies. Investigation of 210 bovine blood samples of Thai cattle from six farms by hematocrit centrifuge technique (HCT) revealed 14 samples with trypanosomes morphologically compatible to *T. theileri*. Additional animals were positive for *T. theileri* by PCR based on the Cathepsin L-like sequence (TthCATL-PCR) despite negative by HCT, indicating cryptic infections. Results revealed a prevalence of 26 ± 15% (95% CI) of *T. theileri* infection. Additionally, 12 samples positive for *T. theileri* were detected in cattle from other 11 farms. From a total of 30 blood samples positive by HCT and/or PCR from 17 farms, seven were characterized to evaluate the genetic polymorphism of *T. theileri* through sequence analysis of PCR-amplified CATL DNA sequences. All CATL sequences of *T. theileri* from Thai cattle clustered with sequences of the previously described phylogenetic lineages TthI and TthII, supporting only two major lineages of *T. theileri* in cattle around the world. However, 11 of the 29 CATL sequences analyzed showed to be different, disclosing an unexpectedly large polymorphic genetic repertoire, with multiple genotypes of *T. theileri* not previously described in other countries circulating in Thai cattle.

1. Introduction

Trypanosoma (*Megatrypanum*) *theileri* is a parasite of cattle transmitted by tabanids that is highly prevalent around the world (Hoare, 1972; Wells, 1976; Böse and Heister, 1993; Rodrigues et al., 2006, 2010a,b). *T. theileri* induces chronic and cryptic infections and is potentially pathogenic for animals under severe physical and nutritional stress, for newborn and pregnant cows, and when associated with concurrent infectious diseases. Infection of *T. theileri* persists for a long time without any clinical signs. The depression of the immune system is thought to allow for increased parasitemia and dispersion of *T. theileri* through several organs and the central nervous system (Ward et al., 1984; Seifi, 1995; Braun et al., 2002; Villa et al., 2008).

There are numerous records of *T. theileri* in cattle. *T. theileri*, usually detected incidentally during cell culture, has been reported in Europe (France, Germany, England, Scotland, Belgium, Italy and Spain), North America (USA, Canada), South America (Brazil) and Asia (Korea and Bangladesh) (Wells, 1976; Schlafer, 1979; Samad and Shahidullah, 1985; Kennedy, 1988; Verloo et al., 2000; Greco et al., 2000; Villa et al., 2008). However, very few isolates derived from cultures have been molecularly

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characterized (Rodrigues et al., 2006, 2010a,b; Hamilton et al., 2009; Lee et al., 2010).

Besides cattle, trypanosomes morphologically indistinguishable from *T. theileri* have been described in buffaloes, sheep, goats and wild ruminants (Rodrigues et al., 2006; Gibson et al., 2010). Trypanosomes from bovids and cervids formed the clade *T. theileri* in phylogenetic trees, which corresponds to the subgenus *T.* (*Megatrypanum*), a taxon comprised solely of trypanosomes from ruminants (Rodrigues et al., 2006, 2010a,b). The sequencing of polymorphic internal transcribed spacer of ribosomal DNA (ITS rDNA), spliced leader (SL) and CATL genes disclosed two phylogenetic lineages, TthI and TthII, and five genotypes of *T. theileri*. Phylogenetic analysis clustered together all *T. theileri* isolates from cattle so far examined, from North and South America, Europe and Asia. Analysis of a large collection of Brazilian cattle isolates from distant regions demonstrated that the lineage TthI includes three genotypes, IA/B/C, whereas TthII comprises two genotypes, IIA and IIB. Genotype ThIA was exclusively found in South American water buffalo and it is closely related to IB/IC cattle genotypes, which have been reported in USA, Japan and Korea. TthII genotypes have been found in Brazil (IIA/B), Germany and Scotland (IIB) (Rodrigues et al., 2006, 2010a,b; Hamilton et al., 2009; Lee et al., 2010).

Because only one isolate was so far characterized from each country except Brazil, diversity and phylogeographical patterns of *T. theileri* circulating in cattle around the world are far from understood. Molecular studies of *T. theileri* are limited by the low levels of parasitemia in ruminant hosts as indicated by rare parasites in blood smears and HCT despite positive hemocultures (Rodrigues et al., 2003, 2006, 2010a,b). Although no serological diagnostic method has been developed specifically for *T. theileri*, a lack of cross-reactivity has been demonstrated for methods used to diagnosis *T. evansi*, *T. congolense*, *T. vivax* and *T. b. brucei* (Desquesnes et al., 2009). *T. theileri* can be distinguished from other trypanosomes by PCR-amplification of ITS rDNA, SL and CATL sequences (Desquesnes et al., 2001; Rodrigues et al., 2006, 2010a,b).

Analysis of the CATL sequences distinguished lineages and genotypes of *T. theileri* (Rodrigues et al., 2006, 2010b). However, these scarce studies are insufficient to understand the population structure and the determinants shaping the genetic diversity and relationships of isolates of *T. theileri*. A recent report mentioned the presence of *T. theileri* in dairy cattle in Thailand (Pruvot et al., 2010). *T. evansi* is the only pathogenic trypanosome that has been reported in Thai ruminants (Indrakamhang, 1998; Desquesnes et al., 2009). In this study, we investigated the prevalence of *T. theileri* in Thai cattle using HCT and PCR-TthCATL diagnostic methods, and the genetic diversity through analysis of CATL DNA sequences.

2. Materials and methods

2.1. Studied area, animals sampled, parasitological examination and DNA preparation

This study was conducted in 6 subdistricts of the Muang district, Buriram province in the northeastern region of Thailand, which is one of the main beef cattle and buffalo breeding areas in the country. In this area, beef cattle are a mixture of Indo-Brazilian, White Lamphun (local) and Charolais. Most farms are of small size, ranging from 2 to 30 head of cattle, and generally have a mixture of cattle and buffaloes.

Blood samples of 210 cattle from six farms were collected from the jugular vein with sodium citrate. HCT was carried out as described previously (Woo, 1969). *T. theileri* was identified based on the large size of the parasite (>60 μm) (Hoare, 1972; Rodrigues et al., 2003). Out of these 210 blood samples, 30 were randomly selected for PCR analysis, in addition to 12 samples positive for trypanosomes by HCT from other 11 neighboring farms. For each of these 42 animals, 200 μl of blood was preserved in 200 μl of ethanol at room temperature for molecular analysis. Ninety five percent confidence interval (95% CI) of *T. theileri* prevalence was estimated in the samples and subsample by parasitological and PCR examination (Ancelle, 2002).

2.2. Diagnosis of T. theileri by PCR-TthCATL and sequence analysis of the amplified DNA

Crude DNA templates from cattle blood samples were used for PCR-TthCATL based on CATL sequence (Rodrigues et al., 2010b). DNA templates were prepared incubating 150 μl of the blood-ethanol mix and 700 μl of lysis buffer (1% SDS, 100 mM EDTA, pH 8.0, 20 mM Tris–HCl, pH 8.0, and 350 μg/mL of proteinase K) at 37 °C for 18 h, and purification using the Wizard DNA Clean-Up System (Promega). DNA fragments amplified (using Taq DNA Polymerase from Fermentas) encodes a ~273 bp sequence of the CATL catalytic domain. All selected samples were tested twice by PCR. For sequence analysis, we selected seven samples that generated the more intense amplified DNA bands, which were purified using Spin-X® kit (Costar®) and cloned using a TA-Cloning® kit (Invitrogen). Sequences of three to five clones of each sample were aligned with those of all established *T. theileri* genotypes (Fig. 1). The sequences determined here have been deposited in GenBank under accession numbers depicted in Fig. 1. Genealogies (Split Networks) were inferred using the Neighbor-Net method as implemented in Splitstree 4.10 (Huson, 1998). Internode supports were estimated by performing 100 bootstrap replicates using the same parameters optimized for network inferences.

2.3. PCR and ELISA assays for the diagnosis of T. evansi

Blood samples were examined using a PCR assay able to detect *T. evansi, T. brucei* ssp. and *T. equiperdum* using primers and reaction conditions previously described (Ventura et al., 2002). Plasma samples were tested by ELISA for *T. evansi* infection according to a previously described method (Desquesnes et al., 2009).

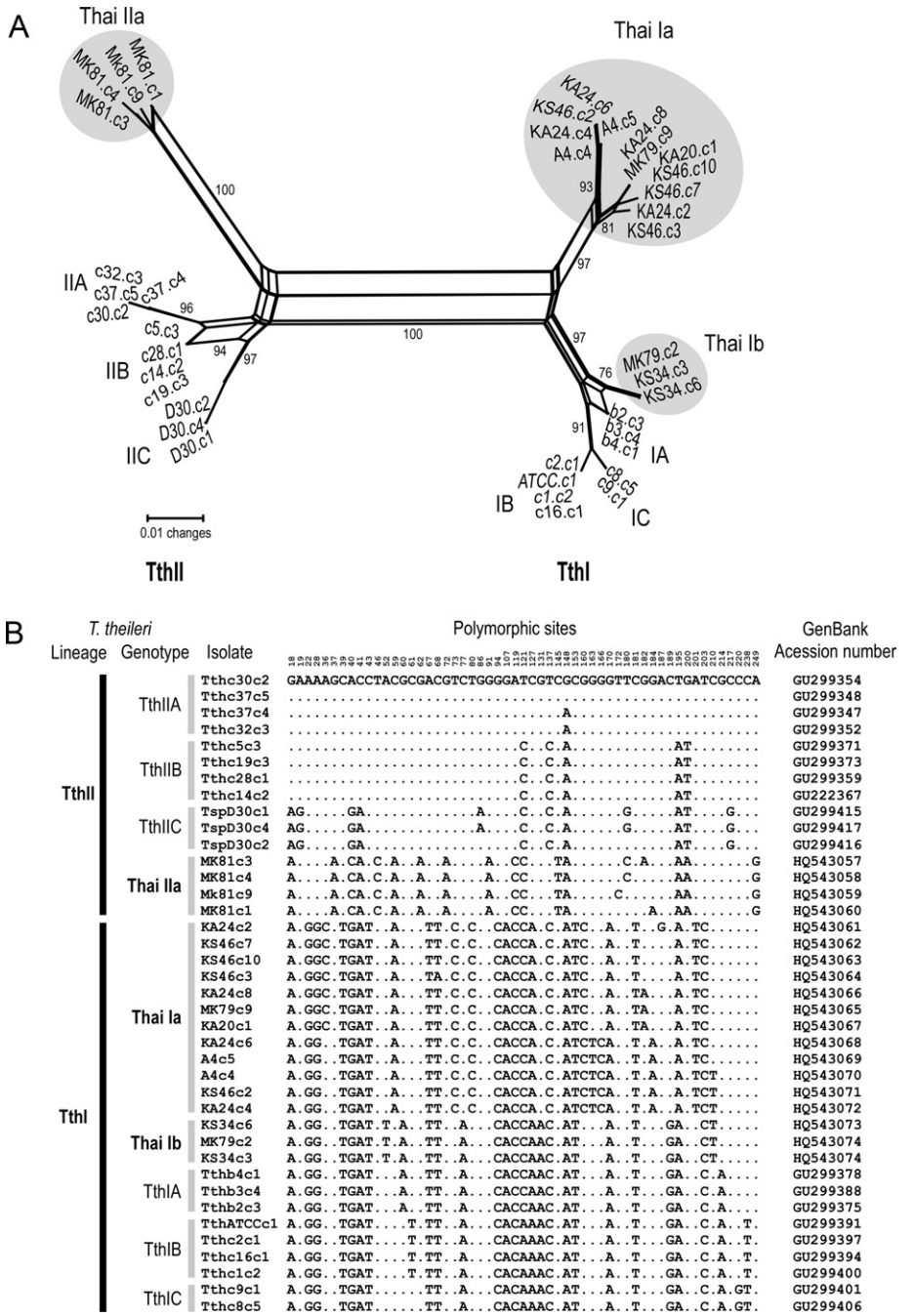

Fig. 1. (A) Network genealogy of sequences from *T. theileri* cathepsin L-like (CATL) genes inferred by the Neighbor-Net method (K2P parameters) showing new genotypes described infecting cattle from Thailand. The new genotypes were separated by large sequence divergences and clustered into the lineages TthI (Thai Ia and Thai Ib) and TthII (Thai IIa) together with previously reported *T. theileri* genotypes from other countries. Numbers correspond to internode support of 100 bootstrap replicates. (B) Polymorphic sites disclosed by the alignment of partial sequences from the catalytic domains of CATL genes evidencing major lineages and genotype diversity within the clade *T. theileri*.

3. Results and discussion

3.1. Prevalence of T. theileri infection in cattle from Thailand as determined by HCT and PCR methods

The large size of the trypanosomes observed in HCT suggested the presence of *T. theileri* in Thai cattle. Out of the 210 cattle blood samples collected in six farms, 14 were positive for *T. theileri* by HCT; parasitological prevalence was then estimated at $7 \pm 3\%$ (95% CI). The scarcity of blood parasites hampered *T. theileri* observation in Giemsa-stained blood smears. Among 30 randomly selected samples from these farms, two samples were positive by HCT (prevalence of $7 \pm 9\%$, 95% CI) and 8 were positive by PCR-TthCATL (preva-

lence of 26 ± 15%, 95% CI). Therefore, the PCR-TthCATL allowed the detection of cryptic *T. theileri* infection in 6 animals negative by HCT, whereas only one sample positive by HCT tested negative for PCR probably due to small amount of blood used for PCR. Considering the low volume of blood tested by HCT and PCR assays, results indicated a high prevalence of *T. theileri* in Thai cattle and the suitability of PCR-TthCATL for epidemiological surveys employing field-collected blood samples preserved in ethanol at room temperature (Rodrigues et al., 2010b). Before this work in Thailand, large surveys of *T. theileri* in cattle using sensitive methods of hemoculture and PCR were only carried out in Brazil (Rodrigues et al., 2003, 2010b).

In addition to cattle blood samples positive for *T. theileri* detected in the survey carried out in this work, 12 samples positive for trypanosomes by HCT and previously selected in surveys done in 11 neighboring farms were confirmed by PCR as infected with *T. theileri*. Therefore, 42 cattle blood samples were tested by PCR-TthCATL, revealing 20 animals positive for *T. theileri* from 17 farms located in Buriram, northeastern Thailand.

3.2. Absence of *T. evansi* in cattle infected with *T. theileri*

In cattle, *T. evansi* is generally considered a mild pathogen resulting in asymptomatic and transient infections. However, a unique epidemiological situation was documented in Asia; acute and chronic signs of disease were observed in cattle and buffalo, with high levels of parasitemia, abortion and death (Reid, 2002). To examine if *T. evansi* infection, which is prevalent in Thai cattle (Indrakamhang, 1998; Desquesnes et al., 2009), could play an important role in course of *T. theileri* infections and vice versa, we investigated the existence of mixed infections with these two species. All *T. theileri* infected animals were negative by PCR for *T. evansi*. Moreover, all the 210 cattle samples were surveyed by ELISA, and none exhibited a positive result for *T. evansi*.

3.3. Diverse and unique repertoire of CATL sequences of *T. theileri* in blood samples from Thailand cattle

Previous analysis of *T. theileri* DNA sequences from primary hemocultures or field-collected blood samples indicated the existence of mixed genotypes in naturally infected Brazilian cattle (Rodrigues et al., 2010a,b). However, in the present study of field-collected blood samples from Thai cattle we have found an unexpected high polymorphism of CATL sequences, either from blood samples of distinct or from the same animal, within and between farms, thus indicating frequent infections with multiple genotypes of *T. theileri*.

To evaluate the genetic diversity among the *T. theileri* isolates found in Thai cattle we compared 29 CATL sequences from 7 blood samples. Ten sequences that showed to be identical to sequences from the clones MK79c9, KA24c4, KA24c6, KS46c7 or KS34c6 (Fig. 1) were removed from the alignment created including sequences (GenBank) of *T. theileri* from other countries (Rodrigues et al., 2010b). Therefore, 19 CATL sequences (11 different) from Thai cattle were used for phylogenetic analysis. Sequences of *T. theileri* from Thai cattle were positioned within TthI or TthII lineages. However, these sequences did not match CATL sequences corresponding to previously determined *T. theileri* genotypes, demonstrating the existence in Thailand of new genotypes not previously described in other countries (Fig. 1).

3.4. Cluster analysis of *T. theileri* CATL sequences from Thailand and other countries

Sequences encoding the catalytic domain of CATL genes have been shown to be phylogenetically informative at the clade, lineage and genotype levels, corroborating the genetic structure within the clade *T. theileri* demonstrated using ITS rDNA and SL genes (Rodrigues et al., 2006, 2010a,b). We previously showed a small polymorphism among cloned CATL encoding genes within each *T. theileri* isolate established in culture, and that sequences from isolates of the same genotype always clustered together (Rodrigues et al., 2010b). However, more than one genotype were detected by PCR-TthCATL when DNA templates were prepared directly from blood samples, primary cultures or from the guts of tabanid flies (Rodrigues et al., 2010b).

In the present study, genealogy using CATL sequences of *T. theileri* found directly in blood samples from Thai cattle, aligned with sequences of isolates from other countries, corroborated the phylogenetic lineages TthI and TthII (Fig. 1B). Despite high polymorphism, most sequences were positioned within TthI in two subclusters (Thai Ia and Thai Ib separated by ~4.7% sequence divergence). These subclusters diverged by ~2.0% and 5.0%, respectively, from the previously established genotypes TthIA and TthIB/C. Only one blood sample (MK79) contained four sequences of ThII that formed the subcluster Thai IIa, which was separated by a large distance (~5.5%) from their closest genotypes TthIIA/B. Thai Ia/Ib and Thai IIa were separated by large distances (average ~11%) and might represent new genotypes so far exclusive of Thailand (Fig. 1A and B).

Although polymorphisms in the CATL sequences used for this study revealed to be enough to define lineages and genotypes, all established genotypes of *T. theileri* were defined using cultured isolates (apparently clonal populations) based on concordant genotyping using three markers: sequences of CATL catalytic domain, ITS rDNA, and SL gene sequences (Rodrigues et al., 2006, 2010a,b). Therefore, more data are necessary before the definitive assigning of new genotypes of *T. theileri* in Thai cattle, as they were determined based on small CATL sequences (273 bp) amplified by PCR-TthCATL. Cultures of *T. theileri* infecting Thai cattle are not available to better characterize the new genotypes described in this study using all these markers.

3.5. Phylogeographical patterns and repertoire of genotypes of *T. theileri* in cattle

According to genealogy of CATL genes described here, sequences of *T. theileri* from Thai cattle predominantly belonged to the TthI lineage and differed from TthI sequences of *T. theileri* found in cattle from Brazil, USA

and Japan. The minority of CATL sequences from Thai isolates that clustered into the TthII lineage differed from TthII genotypes from Brazil, Germany and Scotland. The characterization of *T. theileri* from Thailand supported only two major lineages of *T. theileri,* TthI and TthII, around the world independent of cattle breeds, vector species, and geographical origin. This is the first wide-ranging comparison of *T. theileri* genotypes from countries (Thailand and Brazil) separated by very large geographic distances and without historic or present activity of cattle exchange. Movement of cattle among distinct regions in Thailand is common, but not the introduction of cattle from distant countries, and artificial insemination is used for breeding. Therefore, geographical isolation of cattle could play an important role in the evolution of genotypes restricted to Thailand, while the intensive internal transit of livestock and abundance of tabanid vectors may be determinant for the high parasite diversity in this country. Most likely, human-mediated dispersion of cattle is the main factor interrupting the spatial structuring of *T. theileri* populations in other countries (Rodrigues et al., 2006, 2010a,b).

Results from this study revealed an unexpectedly high polymorphic CATL sequence repertoire, revealing multiple genotypes of *T. theileri* in field-collected blood samples from Thai cattle; suggesting that some recombination process could be responsible for the genetic diversity of parasite populations. The multiple new genotypes of *T. theileri* infecting cattle in Thailand and never found in other countries strongly suggest that new genotypes are waiting to be discovered in unexplored regions. Further studies are necessary for a comprehensive knowledge of genotypes and to understand the evolutionary forces leading the complex repertoire of *T. theileri* populations throughout the world.

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